

UNIVERSITY OF BIRMINGHAM

University of Birmingham
Research at Birmingham

m6A in mRNA: An ancient mechanism for fine-tuning gene expression

Roignant, Jean-Yves; Soller, Matthias

DOI:

[10.1016/j.tig.2017.04.003](https://doi.org/10.1016/j.tig.2017.04.003)

License:

Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Roignant, J-Y & Soller, M 2017, 'm6A in mRNA: An ancient mechanism for fine-tuning gene expression', *Trends in Genetics*, vol. 33, no. 6, pp. 380-390. <https://doi.org/10.1016/j.tig.2017.04.003>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Eligibility for repository: Checked on 6/4/2017

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

m⁶A in mRNA: An ancient mechanism for fine-tuning gene expression

JEAN-YVES ROIGNANT¹ AND MATTHIAS SOLLER²

¹Laboratory of RNA Epigenetics, Institute of Molecular Biology (IMB), Mainz, 55128, Germany

²School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

Running Title: The role of m⁶A in gene expression

Key Words: Sex lethal, Xist, alternative splicing/polyadenylation, mRNA export, mRNA stability, mRNA translation

Matthias Soller
School of Bioscience
College of Life and Environmental Sciences
University of Birmingham
Edgbaston
Birmingham B15 2TT
Tel #: 0121 414 59 05
FAX #: 0121 414 59 25
m.soller@bham.ac.uk

Jean-Yves Roignant
j.roignant@imb-mainz.de

Trends box

Modifications in mRNA have been known for a long time to be present in animals, plants and viruses, and have now also been detected in bacterial and archeal mRNAs. Systematic analysis of their occurrence and functions, however, was hampered until recently by technical limitations to detect these modifications and by the lack of knowledge of the enzymes involved.

Recent advances in high-throughput sequencing technology in combination with selective chemical and immunological identification of modified nucleotides is now revealing the global topology of many modifications in mRNA.

N⁶-methylated adenosine (m⁶A) is one of the most abundant modifications in mRNA. Recent progress has shed light on its biological functions, adding a new layer of post-transcriptional gene regulation.

Abstract

Modifications in messenger RNA (mRNA) constitute ancient mechanisms to regulate gene expression post-transcriptionally. N⁶-methyladenosine (m⁶A) is the most prominent mRNA modification, which is installed by a large methyltransferase complex (the m⁶A ‘writer’), specifically bound by RNA binding proteins (the m⁶A ‘readers’), but also removed by demethylases (the m⁶A ‘erasers’). m⁶A mRNA modifications have been linked to regulation at multiple steps in mRNA processing. In analogy to the regulation of gene expression by miRNAs,

we propose that the main function of m⁶A is post-transcriptional fine-tuning of gene expression. In contrast to miRNA regulation, which mostly reduces gene expression, we argue that m⁶A provides a fast mean to post-transcriptionally maximise gene expression. Additionally, m⁶A appears to have a second function during developmental transitions by targeting m⁶A-marked transcripts for degradation.

Ancient modifications in RNA

Modified nucleotides were first discovered in transfer RNAs (tRNA) and ribosomal RNAs (rRNA), and these modifications are present in all organisms [1-4]. Some of the same nucleotide modifications were later identified in messenger RNAs (mRNA) of prokaryotes, eukaryotes and viruses, revealing an entire new layer of regulation of gene expression [5-8], which has been termed epitranscriptomics (Box 1) [9].

Recent technological advances in high-throughput sequencing in combination with antibody-enrichment and/or induced nucleotide-specific chemical modifications have accelerated the mapping of modifications both on abundant tRNAs and rRNAs and on the less abundant mRNAs [9-16]. A striking observation of these studies was the prevalence of few modifications in mRNA and their non-random distribution, implying important regulatory functions. Furthermore, they can be dynamic and exhibit changes in distribution or frequency in response to various stresses.

*N*⁶-methyladenosine (*m*⁶A) is the most common internal modification in mRNA and long non-coding RNA [9, 12]. *m*⁶A profiling experiments in higher eukaryotes revealed its conserved enrichment near stop codons, long internal exons and, less commonly, start codons. In addition, *m*⁶A has also been found enriched in ribosome-associated mRNA [17-19]. Genetic loss-of-function studies for *m*⁶A methyltransferases (*m*⁶A writers), *m*⁶A-binding proteins (*m*⁶A readers) and *m*⁶A demethylases (*m*⁶A erasers) (see Box 2 and Figure 1) have highlighted a critical role for the *m*⁶A modification in the control of gene expression in several physiological processes (for recent reviews see [6, 20]).

However, it is still not clear precisely how *m*⁶A affects gene expression and what benefit cells and organisms obtain from this extra regulatory layer. A number of recent studies

characterizing the role of m⁶A in alternative splicing and translational regulation provide insights into the more general function of m⁶A in gene expression at the molecular level [17, 18, 19, 28, 32, 41]. Based on these observations detailed and discussed below, we propose that a main function of m⁶A is to fine-tune gene expression.

Functions of m⁶A in development, cellular differentiation and disease

Insights into the function of m⁶A have come from recent RNAi knock-down and genetic knock-out experiments in cell culture and model organisms. Mutants lacking m⁶A have been identified in several organisms including yeast, plants, flies and mice. In the yeast *Saccharomyces cerevisiae*, only meiotic mRNAs are m⁶A methylated and meiosis can only occur if cells possess the core subunit of the methyltransferase complex **IME4** (Inducer of Meiosis-4, see Box 2) [21, 22]. In the plant *Arabidopsis* and in mice, m⁶A is required for viability [23, 24]. Functions in mammals include roles in embryonic stem cell differentiation, maintaining circadian rhythms and dosage compensation (Box 3) [23, 25-30]. Recent studies in *Drosophila* revealed that m⁶A-devoid flies are viable. However, they show reduced fertility and are flightless, and they also suffer from neurological, sex determination and dosage compensation defects (see below) [19, 31, 32].

Emerging evidence also suggests links between m⁶A modifications of mRNA and cell transformation and cancer. For instance, hypoxia promotes breast cancer progression in part by reducing m⁶A levels via increased expression of the demethylase **ALKBH5** (See glossary) [33]. Likewise, m⁶A also plays roles in glioblastoma stem cell renewal and tumorigenesis [34, 35]. Furthermore, several components of the methyltransferase complex including **METTL3**, **METTL14** and **RBM15** are all highly expressed in myeloid leukemia compared with other

cancers, and their alterations are linked with poor prognosis, suggesting that elevated m⁶A levels might predispose to this cancer type [36, 37]. In addition, the m⁶A methyltransferase complex was shown to have an 8-15 fold higher activity upon cellular transformation [38].

m⁶A in meiosis, sex determination and dosage compensation: A unifying theme?

m⁶A is involved in various aspects of sexual development in yeast, *Drosophila* and eutherian mammals. Although many different mechanisms have evolved for sexual differentiation, common elements such as Doublesex transcription factors have been identified in various organisms such as insects and mammals [39]. The role of m⁶A in meiosis in yeast, sex determination and dosage compensation in *Drosophila* as well as dosage compensation in eutherian mammals might indicate a central function for m⁶A in allowing for evolution of diverse sex determination mechanisms observed in nature.

In *S. cerevisiae*, meiosis requires remodeling of the translome, which includes positive selection by marking transcripts with m⁶A for translation [17]. In *S. pombe*, the YTH domain protein **MmiI** adopted a novel mode to binding RNA in the absence of m⁶A. MmiI then selectively removes meiotic transcripts during vegetative growth via the DSR element specifically present in meiotic transcripts [40, 41]. A role for m⁶A in meiosis in mice has been suggested, but such a role in *Drosophila* seems absent [19, 29, 32]. However, the function of m⁶A in yeast is reminiscent of the situation at the maternal-to-zygotic transition (MZT) shortly after fertilization, where the zygote removes maternal transcripts. In zebrafish, this is in part achieved by m⁶A marking of certain maternal transcripts for decay [42]. The same effect could

also be achieved by favoring translation of zygotic transcripts by analogy with favored translation of meiotic transcript in *S. cerevisiae*.

In *Drosophila* and mammals, m⁶A has key roles in the regulation of dosage compensation, but by different mechanisms. In mammals, m⁶A enhances *Xist* function for X chromosome inactivation, while in *Drosophila* m⁶A enhances ***Sex lethal*** (*Sxl*) splicing to achieve maximal *Sxl* expression [19, 28, 32]. *Sxl* then suppresses dosage compensation in females by inhibiting translation of Male-specific lethal-2 (Msl-2), which is required for up-regulation of transcription from the single male X chromosome.

C. elegans employs an unusual mechanism of dosage compensation, in which expression of both X chromosomes is down-regulated in hermaphrodites by about half, and this might offer a hint as to why it does not need m⁶A. Alternatively, loss of m⁶A could potentially be compensated by other mRNA modifications, or could be linked to trans-splicing prominently present in this organism [43]. When we finally understand how *C. elegans* can live without the m⁶A machinery, this may yield insights into the broader functional relevance of this modification.

m⁶A can affect most aspects of gene expression

Co-localization of m⁶A methyltransferase components and RNA Polymerase II (Pol II) at sites of transcription on *Drosophila* polytene chromosomes suggests that m⁶A methylation occurs whilst pre-mRNA is being transcribed [19]. The presence of m⁶A then influences subsequent events in mRNA processing and function in several ways (see below, Figure 1). These include effects on alternative pre-mRNA splicing, 3'-end processing, nuclear export, translation and mRNA decay

[6]. Other possible functions not yet explored might include transcriptional regulation and control of RNA localization.

Pre-mRNA splicing: In mammals, components of the methyltransferase complex localize to speckles, where splicing occurs [44-46]. Since m⁶A is found in introns and affects alternative splicing of *Sxl* pre-mRNA, installation of m⁶A epimarks must occur co-transcriptionally and before splicing (Figure 1) [12, 19].

Likewise m⁶A now has proven roles in regulation of alternative splicing, but it seems not to have a role in localizing splice sites in large introns [19, 32, 47]. For instance, m⁶A present in alternative exons induces their inclusion by directly recruiting the m⁶A-binding reader YTHDC1 [48]. YTHDC1 then recruits the splicing factor **SRSF3**, promoting exon inclusion, and restricts binding of the exon-skipping factor **SRSF10** [48].

Lack of m⁶A in *Drosophila* mRNAs changes alternative splicing in ~2% of genes, with a strong preference (75%) for alternative splicing in **5'UTRs**. Here, m⁶A directed alternative splicing regulation decreases the number of upstream AUGs in 5'UTRs, suggesting that m⁶A-regulated alternative splicing increases translational of the main ORF [19, 32]. The experimentally measured m⁶A levels after a guanosine in *Drosophila* mRNAs are low [19], but it remains uncertain whether m⁶A in *Drosophila* is preferentially in an AAC context, or might initially be incorporated but then be removed – either by uncharacterized demethylases (see below) or in excised and rapidly degraded introns.

In *Sxl* pre-mRNA in *Drosophila*, m⁶A is present in introns on either side of the male-specific alternatively spliced exon that encodes a premature stop codon [19]. This makes expression of *Sxl* female-specific, so that sex determination can occur. To fully exclude this exon in females, *Sxl* binds on either side in the intron, in the vicinity of the m⁶A sites, and requires

assistance from the m⁶A reader YT521-B for full suppression of exon inclusion to achieve maximum Sxl expression. Besides its role in sex determination, Sxl has a second function in dosage compensation. Here, Sxl is required for very tight translational suppression of *msl-2* in females. In males, Msl-2 directs up-regulation of transcription from the single X chromosome to neutralize the unequal numbers of X chromosomes between males and females [19]. The presence of a trace of Msl-2 in females will trigger dosage compensation and compromise viability. Hence, presence of m⁶A in the Sxl intron ensures by enhancing splicing that the maximum amount of Sxl is made to prevent ectopic dosage compensation in females.

The N⁶-methyl group in adenosine weakens base-pairing, which can alter RNA structure [49]. Such structural changes, also termed **m⁶A switch**, have been associated with increased binding of **hnRNPC** and hnRNPG, and concomitant changes in alternative splicing [50, 51]. Whether m⁶A binding by the YTH domain proteins participate in this regulation, however, remains to be determined.

3'-end processing: Knockout of METTL3 altered polyA site selection, indicating a role for m⁶A in alternative polyadenylation [52, 53]. Removal of m⁶A in human A549 cells switched polyA site choice towards the use of proximal polyA sites in a subset of transcripts resulting in shortened **3'UTRs** [52]. Shorter UTRs are generally associated with higher levels of protein expression and cellular proliferation, while longer UTRs are more prevalent in differentiated cells such as neurons. In human embryonic stem cells and the B-cell lymphoblastoid cell line GM12878, presence of m⁶A in the last exon was associated with the use of proximal polyA sites suggesting cell type specific differences in the interpretation of m⁶A epimarks [53].

Nuclear export: Loss of the ALKBH5 eraser increases m⁶A in mRNA and results in more mRNA in the cytoplasm, suggesting that m⁶A-containing mRNAs are more readily exported

from the nucleus [54]. Lack of m⁶A also resulted in delayed appearance of clock gene transcripts in the cytoplasm resulting in an extension of the daily period [25]. Moreover, m⁶A is required for nuclear export of **HIV-1** transcripts [55, 56].

Regulation of translation: An early observation was that the presence of m⁶A stimulated the translation of mouse *dihydrofolate reductase* mRNA using an *in vitro* rabbit reticulocyte translation system [57]. Consistent with a more general role in regulating translation, m⁶A is enriched in ribosome-associated mRNAs of several species [17-19]. The stimulatory effect on translation in mammals is mediated by binding of YTHDF1 to m⁶A near the stop codon, which then interacts with the translation initiation factor eIF3 [18]. In *S. cerevisiae*, **IME4**, and the m⁶A it introduces, is only present during meiosis, when its function is in remodelling of the translome [17]. Also, METTL3 in mammals can stimulate translation in the cytoplasm, independently of its catalytic domain and m⁶A readers, by interacting with the translation machinery via its N-terminal domain [58]. During HIV-1 infection of cells, m⁶A and its YTHDF readers have also been shown to enhance translation and viral replication [55, 56, 59].

A different role for m⁶A in regulating translation is indicated during *Xenopus* oocyte maturation [60]. Here, m⁶A methylated mRNAs are translated less, but m⁶A does not impact on mRNA stability. This suggests that the role of m⁶A in regulating translation may vary depending on cell type and developmental status.

Another mechanism for the regulation of translation by m⁶A comes into play during stress responses (e.g. to UV or heat shock). Here, preferential m⁶A methylation of the 5'UTR of stress response genes leads to cap-independent translation, which involves nuclear re-localization of YTHDF2 to prevent m⁶A removal by the Fat-mass and obesity (**FTO**) demethylase [61, 62]. FTO has been shown to act primarily on N⁶-methyladenosine that is introduced at the first

position after the cap by a currently unknown methyltransferase and shows preference for m⁶A that is also 2'-O-methylated (m⁶Am) [63].

mRNA decay: m⁶A has also been implicated in regulating mRNA decay. When transcription was halted with actinomycin D in cells lacking m⁶A writer the decay of many mRNAs was slowed [27, 64-66]. In contrast, 80% inhibition of m⁶A methylation by an S-adenosylhomocysteine analogue in HeLa delayed appearance of pulse-labelled mRNA in the cytoplasm, probably due to mRNA processing or export defects, but did not change the average half-life of all mRNAs [67].

In mouse embryonic stem (ES) cells m⁶A targets transcripts mediating pluripotency and lineage commitment for degradation [23, 26]. In addition, the reader YTHDF2 is required for clearance of maternal mRNA at the MZT early in zebrafish embryonic development [42]. These observations argue that m⁶A has a role in promoting mRNA decay, but this function might be restricted to certain cell types such as ES cells or to specific developmental transitions. A mutational analysis of m⁶A in labile transcripts would be required to determine whether m⁶A does indeed target transcripts for degradation.

In mammals, the YTH domain-containing protein YTHDF2 recognizes m⁶A through its YTH C-terminal domain and directs mRNA to cytoplasmic P-bodies by recruiting the **CCR4-NOT deadenylase complex**, initiating deadenylation and degradation of methylated transcripts [65, 68]. Recently, YTHDF3, another cytoplasmic protein, was shown to interact co-operatively with YTHDF2 to accelerate mRNA decay [66]. YTHDF3 in co-operation with a third YTH protein, YTHDF1 also promotes translation of methylated RNAs [66, 69]. It is not yet clear how these different interactions between YTH proteins occur and what controls their selectivity towards m⁶A sites. This is likely to depend on sequence context and may be dynamically regulated.

miRNA processing: Processing of pri-miRNAs has been shown to require m⁶A-facilitated binding of **DGCR8**, which is important for recruitment of DROSHA for nuclear pri-miRNA cleavage. Promoting miRNA processing further requires binding of hnRNPA2B1 to m⁶A in pri-miRNAs [70, 71].

Long non-coding RNAs: A role for m⁶A has been revealed in the long non-coding RNA *Xist*, which is required for dosage compensation in humans. Here, m⁶A in *Xist* RNA promotes heterochromatinization to transcriptionally inactivate one of the X chromosomes [28].

In summary, the role of m⁶A in enhancing alternative splicing, stimulation of nuclear export of mRNAs and translation suggest a broad function in boosting gene expression from pre-set regulation. In addition, m⁶A appears to have a second function in promoting removal and perhaps translational silencing of m⁶A marked transcripts to facilitate developmental transitions as in oocyte development upon commencement of meiosis, in maternal to zygotic transition in early embryogenesis and from stem cell renewal to the initiation of differentiation. All together, these examples suggest a key role for m⁶A in fine-tuning gene expression.

Tweaking the output: Analogy to miRNA regulation, but mostly to get more?

A role for m⁶A in fine-tuning gene expression post-transcriptionally is reminiscent of the role of miRNAs in this process. A fundamental feature of miRNA regulation, however, is down-regulation of gene expression. miRNAs bound by Argonaute proteins affect mRNA stability by directing mRNA cleavage or by inhibiting translation; and there are only a few exceptions to this rule [72-75].

It may therefore be reasonable to suggest that cells contain a mechanism to balance the action of miRNA regulation for buffering fluctuations in gene expression by up-regulating

translation (Figure 2). Such a role of m⁶A to boost gene expression makes biological sense for cells that need to react quickly to developmental signals during cellular differentiation, but also to adapt to environmental changes by altering metabolism.

Meiosis in yeast is induced by starvation. Although starvation stress generally reduces gene expression, meiotic transcripts evade this response by being m⁶A methylated, which in turn enhances their translation [17]. A role for m⁶A in up-regulating translation has also been found in *dihydrofolate reductase* and *p21* transcripts [57, 76]. Likewise, a more general role of m⁶A in enhancing translation is indicated from enrichment of m⁶A on translating ribosomes and a direct interaction of the m⁶A reader YTHDF1 with the translation initiation factor eIF3 [17-19]. Consistent with such a role, viruses such as HIV-1 have exploited this route to enhance production of viral proteins to be ahead of the host response [55, 56, 59].

A further role for m⁶A in promoting translation has been revealed during heat-shock stress. Under such conditions of general shut down-of gene expression, m⁶A in the 5'UTR can direct selective translation of transcripts after de-capping [61, 62]. UV-light induced DNA damage also relies on m⁶A, but whether this involves enhanced translation e.g. of DNA repair enzymes needs to be elaborated [77].

A different role in enhancing protein production has been assigned to N⁶-methyladenosine when in the cap adjacent position. Here, m⁶A and m⁶Am directly counteract miRNA mediated de-capping [63].

Another mechanism for making more protein has been revealed in the *Drosophila* sex determination pathway where the amount of female-specific Sxl is most critical for fully suppressing dosage compensation. Here, m⁶A in the introns flanking an alternatively spliced exon is required to enhance skipping of this exon, whose inclusion results in a truncated non-

functional Sxl protein. In fact, m⁶A present in alternative exons has also been shown to enhance their inclusion likely resulting in higher protein levels of longer isoforms [48].

In human dosage compensation, m⁶A enhances the function of the non-coding RNA *Xist* in inactivating one of the X chromosomes in females. The exact mechanism how m⁶A in *Xist* drives condensation of the chromatin of this X chromosome, however, is not known [28].

In support of such a ubiquitous role of m⁶A in adjusting gene expression, we find methyltransferase complex components associated with most if not all RNA Pol II transcribed genes in *Drosophila* [19]. Since *Drosophila* is weakly viable without m⁶A, this suggests that m⁶A is mostly used for non-vital aspects of gene expression to increase fitness and adaptability. Since many miRNA mutants also display very mild phenotypes (reviewed in [75]) a main role for m⁶A in *Drosophila* to counteract miRNA regulation appears possible.

The presence of demethylases remains to be demonstrated in *Drosophila*, but they could be absent. In addition, it is also not clear whether m⁶A affects mRNA stability and pri-miRNA processing in *Drosophila*.

Functional antagonism between m⁶A and miRNAs is further suggested from Argonaute binding sites which overlap with m⁶A sites in mRNA 3'UTR of mouse brains [44]. However, there are likely exceptions to a simple model whereby m⁶A's main role is in enhancing gene expression to counteract miRNAs. Notably, mammals have expanded m⁶A regulation to vital processes and also increased the number of YTH reader proteins compared to *Drosophila*. Likewise, m⁶A is required for efficient processing of pri-miRNA and thus m⁶A supports miRNA mediated down-regulation of gene expression. Further regulatory interplay is indicated from miRNAs that impact on m⁶A levels in transcripts of YTH domain proteins to affect their translation [52, 78, 79]. Likewise, m⁶A has also been shown to enhance mRNA decay in some

cell types [27, 42, 64-66]. In zebrafish, m⁶A is further assisted by miR-430 in clearance of maternal transcripts at the MZT [42].

It remains to be determined, however, whether m⁶A is differentially recognized by YTH domain proteins for transcript-specific fates in different cell-types or whether m⁶A is used in a cell type-specific manner to either direct translational enhancement or mRNA decay. Potentially, transcript specific outcomes could be generated through interaction of YTH proteins with other RNA binding proteins such as Sxl or related ELAV/Hu family proteins [12, 19, 80].

In addition, demethylases can remove m⁶A epimarks, but this is not simply a cytoplasmic pathway to remove nuclear installed m⁶A as ALKBH5 localizes to nuclear speckles and FTO shuttles between the nucleus and the cytoplasm [54, 63].

Concluding Remarks

The novel field of epitranscriptomics has yielded fundamental progress in defining the topology of mRNA modifications genome-wide – and especially of m⁶A, the most abundant modification. Further technological advances will be needed before we can measure low and dynamic changes in m⁶A [81], maybe by harnessing new sequencing technologies that allow the determination of mRNA modifications in single molecules.

Now, biological functions for this enigmatic m⁶A modification are emerging through more mechanistic analysis revealing an ancient mechanism to fine-tune gene expression.

Much remains to be learned before we can understand how the dynamic regulation of the co-transcriptional installation of m⁶A methyl marks is dynamically instructed, how m⁶A impacts on downstream mRNA processing events and how its mis-regulation results in human disease (See Outstanding Questions).

Outstanding question box

- Is m⁶A introduced co-transcriptionally, and if so by what mechanism?
- How are putative m⁶A methylation sites recognized, and why are only some of them methylated?
- How is the emplacement of an m⁶A epimark regulated?
- How is m⁶A recognized, in various contexts, by the various reader proteins?
- Does m⁶A modification target individual mRNAs to specific fates – for example, to be translated faster or to be degraded?
- Does m⁶A have a role in dosage compensation in organisms other than humans and *Drosophila*?

Box 1: Epitranscriptomics (greek “epi”: on top of)

Modification of nucleotides in mRNA adds additional information that is not encoded in the mRNA or DNA sequence. The novel field of epitranscriptomics examines where modified nucleotides are present in mRNA, how they are emplaced, read and removed (by ‘writers’, ‘readers’ and ‘erasers’) and how they may modulate mRNA structure [82] (see Box 2). Methyl epimarks are metabolically dynamic, suggesting a highly regulated process. It is possible that modified nucleotides in mRNA might be inherited epigenetically – e.g. in RNA viruses or from the mother – but no such mechanism has yet been found.

Box 2: The m⁶A writers, readers and erasers.

Writers: Methylation of A bases to m⁶A in mRNAs is catalysed by a methyltransferase complex that includes the catalytic subunit METHylTransferase Like 3 (**METTL3**, also called MT-A70, MTA or IME4), METHylTransferase Like 14 (**METTL14**), which seems to be catalytically inactive, Wilms Tumor 1 Associated Protein (**WTAP**, also called FI(2)d), KIAA1429 (also called Virilizer) and RNA Binding Motif 15 (**RBM15**, also called Spenito) [19, 22, 27, 32, 46, 64, 83-87]. Core m⁶A methyltransferase complex components (METTL3, and METTL14, WTAP and Vir) are highly conserved in most eukaryotes, but are absent from the fission yeast *S. pombe* and the nematode *C. elegans* [88, 89], suggesting that m⁶A on mRNA is not present in these organisms.

Readers: YTH domain-containing proteins have recently been identified as readers of m⁶A marks on mRNA. They display a 10-50-fold higher affinity for m⁶A methylated mRNA than for unmethylated mRNA [19, 90-93]. Animals have two families of YTH proteins. One has two members in humans (YTHDC1 and YTHDC2) and one in *Drosophila* (YT521-B), and the other has three members in humans (YTHDF1/2/3) and one in *Drosophila* (CG6422). Plants have many more (13 in *Arabidopsis*) [88]. In the nematode *C. elegans*, YTH proteins are also absent [88].

Erasers: m⁶A can be reverted to adenosine by the m⁶A RNA demethylase ALKBH5 in mammals, but whether demethylases exist in lower eukaryotes remains to be determined [54]. A second demethylase from mammals, Fat Mass and Obesity-associated protein (FTO) was initially thought to play a similar function, but a recent report suggests that its *in vivo* activity is rather dedicated towards the methylation present when the first cap adjacent nucleotide is an adenosine. The enzyme placing this m⁶A modification is not yet known [54, 63, 94, 95].

Box 3: Dosage compensation

Dosage compensation describes the adjustment of gene expression for the unequal numbers of sex chromosomes in males and females: Aneuploidy of sex chromosomes would otherwise be lethal. Different organisms use a variety of mechanisms both for sex determination and for dosage compensation. In humans, one female X chromosome is inactivated; in *Drosophila*, the male X chromosome is up-regulated to transcribe twice as much; and in *Caenorhabditis*, both X chromosomes in hermaphrodites are down-regulated [19, 28].

Glossary

(see Box 2, main text for additional explanations of gene and protein functions)

3'-UTR (3'-untranslated region)

region of the mRNA after the stop codon of the coding sequence

5'-UTR (5'-untranslated region)

region of the mRNA before the start codon of the coding sequence

ALKBH5 (ALKB Homolog 5)

m⁶A mRNA demethylase that removes the methyl group associated with N⁶-methyladenosine

CCR4-NOT complex

conserved multi-subunit complex involved in mRNA deadenylation

DGCR8

subunit of the microprocessor complex that mediates the biogenesis of miRNA from the primary microRNA transcript

DSR (Determinant of selective removal)

cis-regulatory element that targets yeast meiotic specific mRNA for decay by the exosome via Mmi1 recruitment

FTO (Fat mass and obesity associated protein)

m⁶A mRNA demethylase that removes the methyl group of N⁶-methyladenosine present at the first position after the cap

HIV-1 (Human Immunodeficiency virus)

Lentivirus (subclass of retrovirus) that causes the acquired immunodeficiency syndrome (AIDS)

hnRNP C (heterogeneous nuclear Ribonucleoprotein C)

RNA binding protein that binds m⁶A-containing RNA due to changes in local structure of the mRNA induced by m⁶A

IME4 (Inducer of Meiosis-4)

component of the methyltransferase complex, also named METTL3

m⁶A-switch

alteration of the local structure of the mRNA induced by m⁶A

Mmi1

YTH domain-containing protein, which lost its ability to recognize m⁶A and directs yeast meiotic mRNAs to degradation via binding to the DRS.

METTL3 (Methyltransferase like 3)

methyltransferase involved in m⁶A deposition on mRNA, also named MTA-70, MTA and IME4.

METTL14 (Methyltransferase like 14)

component of the m⁶A methyltransferase complex that serves as a support protein for METTL3, but seems catalytically inactive.

RBM15 (RNA Binding Motif Protein 15)

Member of the Split-end family of protein and component of the m⁶A methyltransferase complex that recruits the methylosome to its target mRNAs

Sxl (Sex lethal)

master regulator of the sex determination and dosage compensation pathways in *Drosophila*.

SRSF3 (Serine and Argine Rich Splicing Factor 3)

splicing factor recruited to pre-mRNA near m⁶A sites via the m⁶A reader YTHDC1, resulting in exon inclusion

SRSF10 (Serine and Arginine Rich Splicing Factor 10)

Splicing factor that interacts with the m⁶A reader YTHDC1, preventing its binding to mRNA close to m⁶A-containing regions, leading to exon skipping.

WTAP (Wilms Tumor 1 Associated Protein)

subunit of the methyltransferase complex that stabilizes the integrity of the heterodimer METTL3/METTL14.

YTH proteins

Class of proteins that specifically recognize m⁶A in mRNA

Acknowledgments

We apologize to colleagues whose work was not cited owing to space limitations. We thank I. Haussmann, M. Helm and R. Michell for comments on the manuscript. For this work, we acknowledge funding from the European COST action (CA16120) to JY. Roignant and from the Biotechnology and Biological Sciences Research Council (BBSRC) to M. Soller.

References

1. Cohn WE, V.E. (1951) Nucleoside-5'-Phosphates from Ribonucleic Acid. *Nature* 167, 483-484
2. Davis, F.F., and Allen, F.W. (1957) Ribonucleic acids from yeast which contain a fifth nucleotide. *J Biol Chem* 227, 907-915
3. Motorin, Y., and Helm, M. (2011) RNA nucleotide methylation. *Wiley Interdiscip Rev RNA* 2, 611-631
4. Machnicka, M.A., *et al.* (2013) MODOMICS: a database of RNA modification pathways--2013 update. *Nucleic Acids Res* 41, D262-267
5. Gilbert, W.V., *et al.* (2016) Messenger RNA modifications: Form, distribution, and function. *Science* 352, 1408-1412
6. Zhao, B.S., *et al.* (2017) Post-transcriptional gene regulation by mRNA modifications. *Nat Rev Mol Cell Biol* 18, 31-42
7. Deng, X., *et al.* (2015) Widespread occurrence of *N*⁶-methyladenosine in bacterial mRNA. *Nucleic Acids Res* 43, 6557-6567
8. Marbaniang, C.N., and Vogel, J. (2016) Emerging roles of RNA modifications in bacteria. *Curr Opin Microbiol* 30, 50-57

9. Meyer, K.D., *et al.* (2012) Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell* 149, 1635-1646
10. Carlile, T.M., *et al.* (2014) Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature* 515, 143-146
11. Delatte, B., *et al.* (2016) RNA biochemistry. Transcriptome-wide distribution and function of RNA hydroxymethylcytosine. *Science* 351, 282-285
12. Dominissini, D., *et al.* (2012) Topology of the human and mouse m⁶A RNA methylomes revealed by m⁶A-seq. *Nature* 485, 201-206
13. Dominissini, D., *et al.* (2016) The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature* 530, 441-446
14. Li, X., *et al.* (2016) Transcriptome-wide mapping reveals reversible and dynamic N¹-methyladenosine methylome. *Nat Chem Biol* 12, 311-316
15. Li, X., *et al.* (2015) Chemical pulldown reveals dynamic pseudouridylation of the mammalian transcriptome. *Nat Chem Biol* 11, 592-597
16. Schwartz, S., *et al.* (2014) Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell* 159, 148-162
17. Bodi, Z., *et al.* (2015) Yeast m⁶A Methylated mRNAs Are Enriched on Translating Ribosomes during Meiosis, and under Rapamycin Treatment. *PLoS One* 10, e0132090
18. Wang, X., *et al.* (2015) N⁶-methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell* 161, 1388-1399
19. Haussmann, I.U., *et al.* (2016) m⁶A potentiates Sxl alternative pre-mRNA splicing for robust *Drosophila* sex determination. *Nature* 540, 301-304

20. Wang, Y., and Zhao, J.C. (2016) Update: Mechanisms Underlying N^6 -Methyladenosine Modification of Eukaryotic mRNA. *Trends Genet* 32, 763-773
21. Clancy, M.J., *et al.* (2002) Induction of sporulation in *Saccharomyces cerevisiae* leads to the formation of N^6 -methyladenosine in mRNA: a potential mechanism for the activity of the IME4 gene. *Nucleic Acids Res* 30, 4509-4518
22. Schwartz, S., *et al.* (2013) High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. *Cell* 155, 1409-1421
23. Geula, S., *et al.* (2015) Stem cells. m^6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science* 347, 1002-1006
24. Zhong, S., *et al.* (2008) MTA is an Arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *Plant Cell* 20, 1278-1288
25. Fustin, J.M., *et al.* (2013) RNA-methylation-dependent RNA processing controls the speed of the circadian clock. *Cell* 155, 793-806
26. Batista, P.J., *et al.* (2014) m^6A RNA modification controls cell fate transition in mammalian embryonic stem cells. *Cell Stem Cell* 15, 707-719
27. Wang, Y., *et al.* (2014) N^6 -methyladenosine modification destabilizes developmental regulators in embryonic stem cells. *Nat Cell Biol* 16, 191-198
28. Patil, D.P., *et al.* (2016) m^6A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* 537, 369-373
29. Klungland, A., *et al.* (2016) Reversible RNA modifications in meiosis and pluripotency. *Nat Methods* 14, 18-22
30. Frye, M., and Blanco, S. (2016) Post-transcriptional modifications in development and stem cells. *Development* 143, 3871-3881

31. Hongay, C.F., and Orr-Weaver, T.L. (2011) Drosophila Inducer of MEiosis 4 (IME4) is required for Notch signaling during oogenesis. *Proc Natl Acad Sci U S A* 108, 14855-14860
32. Lence, T., *et al.* (2016) m⁶A modulates neuronal functions and sex determination in Drosophila. *Nature* 540, 242-247
33. Zhang, C., *et al.* (2016) Hypoxia-inducible factors regulate pluripotency factor expression by ZNF217- and ALKBH5-mediated modulation of RNA methylation in breast cancer cells. *Oncotarget* 7, 64527-64542
34. Cui, Q., *et al.* (2017) m⁶A RNA Methylation Regulates the Self-Renewal and Tumorigenesis of Glioblastoma Stem Cells. *Cell Rep* 18, 2622-2634
35. Zhang, S., *et al.* (2017) m⁶A Demethylase ALKBH5 Maintains Tumorigenicity of Glioblastoma Stem-like Cells by Sustaining FOXM1 Expression and Cell Proliferation Program. *Cancer Cell*
36. Jaffrey, S.R., and Kharas, M.G. (2017) Emerging links between m⁶A and misregulated mRNA methylation in cancer. *Genome Med* 9, 2
37. Kwok, C.T., *et al.* (2017) Genetic alterations of m⁶A regulators predict poorer survival in acute myeloid leukemia. *J Hematol Oncol* 10, 39
38. Tuck, M.T., *et al.* (1996) Elevation of internal 6-methyladenine mRNA methyltransferase activity after cellular transformation. *Cancer Lett* 103, 107-113
39. Raymond, C.S., *et al.* (1998) Evidence for evolutionary conservation of sex-determining genes. *Nature* 391, 691-695
40. Wang, C., *et al.* (2016) A novel RNA-binding mode of the YTH domain reveals the mechanism for recognition of determinant of selective removal by Mmi1. *Nucleic Acids Res* 44, 969-982

41. Harigaya, Y., *et al.* (2006) Selective elimination of messenger RNA prevents an incidence of untimely meiosis. *Nature* 442, 45-50
42. Zhao, B.S., *et al.* (2017) m6A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. *Nature*
43. Allen, M.A., *et al.* A global analysis of *C. elegans* trans-splicing. *Genome Res* 21, 255-264
44. Bokar, J.A., *et al.* (1997) Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N^6 -adenosine)-methyltransferase. *RNA* 3, 1233-1247
45. Uranishi, H., *et al.* (2009) The RNA-binding motif protein 15B (RBM15B/OTT3) acts as cofactor of the nuclear export receptor NXF1. *J Biol Chem* 284, 26106-26116
46. Ping, X.L., *et al.* (2014) Mammalian WTAP is a regulatory subunit of the RNA N^6 -methyladenosine methyltransferase. *Cell Res* 24, 177-189
47. Soller, M. (2006) Pre-messenger RNA processing and its regulation: a genomic perspective. *Cell Mol Life Sci* 63, 796-819
48. Xiao, W., *et al.* (2016) Nuclear m⁶A Reader YTHDC1 Regulates mRNA Splicing. *Mol Cell* 61, 507-519
49. Roost, C., *et al.* (2015) Structure and thermodynamics of N^6 -methyladenosine in RNA: a spring-loaded base modification. *J Am Chem Soc* 137, 2107-2115
50. Liu, N., *et al.* (2015) N^6 -methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. *Nature* 518, 560-564
51. Liu, N., *et al.* (2017) N^6 -methyladenosine alters RNA structure to regulate binding of a low-complexity protein. *Nucleic Acids Res*

52. Ke, S., *et al.* (2015) A majority of m⁶A residues are in the last exons, allowing the potential for 3' UTR regulation. *Genes Dev* 29, 2037-2053
53. Molinie, B., *et al.* (2016) m⁶A -LAIC-seq reveals the census and complexity of the m(6)A epitranscriptome. *Nat Methods* 13, 692-698
54. Zheng, G., *et al.* (2013) ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell* 49, 18-29
55. Kennedy, E.M., *et al.* (2016) Posttranscriptional m⁶A Editing of HIV-1 mRNAs Enhances Viral Gene Expression. *Cell Host Microbe* 19, 675-685
56. Lichinchi, G., *et al.* (2016) Dynamics of the human and viral m⁶A RNA methylomes during HIV-1 infection of T cells. *Nat Microbiol* 1, 16011
57. Heilman, K.L., *et al.* (1996) Internal 6-methyladenine residues increase the in vitro translation efficiency of dihydrofolate reductase messenger RNA. *Int J Biochem Cell Biol* 28, 823-829
58. Lin, S., *et al.* (2016) The m⁶A Methyltransferase METTL3 Promotes Translation in Human Cancer Cells. *Mol Cell* 62, 335-345
59. Tirumuru, N., *et al.* (2016) N⁶-methyladenosine of HIV-1 RNA regulates viral infection and HIV-1 Gag protein expression. *Elife* 5
60. Qi, S.T., *et al.* (2016) N⁶-Methyladenosine Sequencing Highlights the Involvement of mRNA Methylation in Oocyte Meiotic Maturation and Embryo Development by Regulating Translation in *Xenopus laevis*. *J Biol Chem* 291, 23020-23026
61. Meyer, K.D., *et al.* (2015) 5' UTR m⁶A Promotes Cap-Independent Translation. *Cell* 163, 999-1010

62. Zhou, J., *et al.* (2015) Dynamic m⁶A mRNA methylation directs translational control of heat shock response. *Nature* 526, 591-594
63. Mauer, J., *et al.* (2017) Reversible methylation of m⁶Am in the 5' cap controls mRNA stability. *Nature* 541, 371-375
64. Schwartz, S., *et al.* (2014) Perturbation of m⁶A writers reveals two distinct classes of mRNA methylation at internal and 5' sites. *Cell Rep* 8, 284-296
65. Wang, X., *et al.* (2014) N⁶-methyladenosine-dependent regulation of messenger RNA stability. *Nature* 505, 117-120
66. Shi, H., *et al.* (2017) YTHDF3 facilitates translation and decay of N⁶-methyladenosine-modified RNA. *Cell Res*
67. Camper, S.A., *et al.* (1984) Effect of undermethylation on mRNA cytoplasmic appearance and half-life. *Mol Cell Biol* 4, 538-543
68. Du, H., *et al.* (2016) YTHDF2 destabilizes m⁶A-containing RNA through direct recruitment of the CCR4-NOT deadenylase complex. *Nat Commun* 7, 12626
69. Li, A., *et al.* (2017) Cytoplasmic m⁶A reader YTHDF3 promotes mRNA translation. *Cell Res*
70. Alarcon, C.R., *et al.* (2015) HNRNPA2B1 Is a Mediator of m⁶A-Dependent Nuclear RNA Processing Events. *Cell* 162, 1299-1308
71. Alarcon, C.R., *et al.* (2015) N⁶-methyladenosine marks primary microRNAs for processing. *Nature* 519, 482-485
72. Jiao, A.L., and Slack, F.J. (2014) RNA-mediated gene activation. *Epigenetics* 9, 27-36
73. Ameres, S.L., and Zamore, P.D. (2013) Diversifying microRNA sequence and function. *Nat Rev Mol Cell Biol* 14, 475-488

74. Lee, S., and Vasudevan, S. (2012) Post-transcriptional stimulation of gene expression by microRNAs. *Adv Exp Med Biol* 768, 97-126
75. Ebert, M.S., and Sharp, P.A. (2012) Roles for microRNAs in conferring robustness to biological processes. *Cell* 149, 515-524
76. Li, Q., *et al.* (2017) NSUN2-mediated m⁵C Methylation and METTL3/METTL14-mediated m⁶A Methylation Cooperatively Enhance p21 Translation. *J Cell Biochem*
77. Xiang, Y., *et al.* (2017) RNA m⁶A methylation regulates the ultraviolet-induced DNA damage response. *Nature* 543, 573-576
78. Li, A., *et al.* Cytoplasmic m⁶A reader YTHDF3 promotes mRNA translation. *Cell Res*
79. Chen, T., *et al.* m⁶A RNA methylation is regulated by microRNAs and promotes reprogramming to pluripotency. *Cell Stem Cell* 16, 289-301
80. Zaharieva, E., *et al.* (2015) Concentration and Localization of Coexpressed ELAV/Hu Proteins Control Specificity of mRNA Processing. *Mol Cell Biol* 35, 3104-3115
81. Helm, M., and Motorin, Y. (2017) Detecting RNA modifications in the epitranscriptome: predict and validate. *Nat Rev Genet*
82. Saletore, Y., *et al.* (2012) The birth of the Epitranscriptome: deciphering the function of RNA modifications. *Genome Biol* 13, 175
83. Liu, J., *et al.* (2014) A METTL3-METTL14 complex mediates mammalian nuclear RNA N⁶-adenosine methylation. *Nat Chem Biol* 10, 93-95
84. Horiuchi, K., *et al.* (2013) Identification of Wilms' tumor 1-associating protein complex and its role in alternative splicing and the cell cycle. *J Biol Chem* 288, 33292-33302
85. Wang, P., *et al.* (2016) Structural Basis for Cooperative Function of Mettl3 and Mettl14 Methyltransferases. *Mol Cell* 63, 306-317

86. Wang, X., *et al.* (2016) Structural basis of N^6 -adenosine methylation by the METTL3-METTL14 complex. *Nature* 534, 575-578
87. Sledz, P., and Jinek, M. (2016) Structural insights into the molecular mechanism of the m^6A writer complex. *Elife* 5
88. Dezi, V., *et al.* (2016) Nucleotide modifications in messenger RNA and their role in development and disease. *Biochem Soc Trans* 44, 1385-1393
89. Lence, T., *et al.* (2017) A fly view on the roles and mechanisms of the m^6A mRNA modification and its players. *RNA Biol*, 0
90. Theler, D., *et al.* (2014) Solution structure of the YTH domain in complex with N^6 -methyladenosine RNA: a reader of methylated RNA. *Nucleic Acids Res* 42, 13911-13919
91. Wang, X., and He, C. (2014) Reading RNA methylation codes through methyl-specific binding proteins. *RNA Biol* 11, 669-672
92. Xu, C., *et al.* (2014) Structural basis for selective binding of m^6A RNA by the YTHDC1 YTH domain. *Nat Chem Biol* 10, 927-929
93. Zhu, T., *et al.* (2014) Crystal structure of the YTH domain of YTHDF2 reveals mechanism for recognition of N^6 -methyladenosine. *Cell Res* 24, 1493-1496
94. Jia, G., *et al.* (2011) N^6 -methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol* 7, 885-887
95. Fu, Y., *et al.* (2013) FTO-mediated formation of N^6 -hydroxymethyladenosine and N^6 -formyladenosine in mammalian RNA. *Nat Commun* 4, 1798

Figure legends

Figure 1. The writers, readers and erasers of m⁶A.

The m⁶A writer complex is composed of at least five proteins, including the catalytic subunit METTL3. METTL14 serves as structural support for METTL3, while WTAP stabilizes the core complex. RBM15 helps in recruiting the complex to its target sites. The molecular function of Virilizer is currently unknown.

Co-localization of m⁶A writers with RNA Pol II on *Drosophila* polytene chromosomes suggests co-transcriptional installation of m⁶A epimarks [19]. ALKBH5 and FTO are two demethylases that remove the methyl group from m⁶A and m⁶Am (2'-*O*-ribosemethylated m⁶A), respectively. m⁶A readers include hnRNPA2B1 and members of the YTH-domain family of proteins. These factors mediate the function of m⁶A in several processes including X-chromosome inactivation, pre-mRNA splicing and 3'end processing, mRNA export, translation and mRNA decay. Other possible functions not yet explored might include transcriptional regulation and RNA localization.

Figure 2: Roles of m⁶A in regulating gene expression.

Model that depicts opposing roles of m⁶A and miRNA in fine-tuning gene expression during normal mammalian development and adult homeostasis. While miRNA mainly down-regulates gene expression m⁶A in contrast maximizes it (black arrows).

However, at specific developmental transitions or/and cell types m⁶A can also reduce gene expression (dashed arrow). Likewise, in very few cases, miRNA was shown to increase gene expression (thin dash arrow). m⁶A can also directly facilitate miRNA biogenesis, whereas a

specific miRNA was shown to influence m⁶A levels via regulation of the m⁶A reader YTHDF2 (double dashed arrow). The situation in *Drosophila* is similar but less complex (see main text).



